IMMUNOLOGICAL STUDIES ON GLOBULAR AND ELONGATED FORMS OF ELECTRIC EEL ACETYLCHOLINESTERASE. EFFECTS OF HYDROLYTIC ENZYMES

François RIEGER
Laboratoire de Neurobiologie, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France

and

Philippe BENDA, Annie BAUMAN and Jean ROSSIER
Laboratoire de Biologie Moléculaire, Collège de France, 11, Place Marcelin Berthelot, 75005 Paris, France

Received 15 February 1973

1. Introduction

It is known that several forms of acetylcholinesterase (AchE) have been found, differing by their sedimentation constant and by their structure [1, 2]: globular forms (11.8 S) and elongated forms which electron microscopy showed to be composed of a rod-like structure (called the "tail") and a cluster of varying numbers (one to three) tetramers (called the "head"). These elongated forms had been separated into A (8.5 S), C (14.2 S) and D (18.4 S). Antibodies to AchE had already been produced [3, 4] with purified enzyme (globular form) prepared according to Leuzinger and Baker [5].

We have now produced, in rabbits, antibodies to the globular and elongated forms of AchE, in order to compare the reactivity of each serum to homologous and heterologous antigens. We found that the elongated forms were more reactive than the globular forms with each antiserum tested, presumably because of a "squeezing" effect only found in the latter forms.

However, the presence of non-protidic components having been shown in the AchE molecule [6], we investigated (by microcomplement fixation assays) the effect of phospholipid and polysaccharide hydrolytic enzymes on the various forms of AchE, in search of a distinct immunological feature related to minute conformational changes.

After treatment with neuraminidase, we observed a progressive decrease in the affinity of the antiserum for the D and C forms, and no effect on the globular forms. This would mean that some polysaccharides are important for the structure of the elongated forms.

2. Material and methods

2.1. *Preparation of antigens*

Elongated A, C and D forms of AchE were prepared from saline homogenates of fresh electric organ, and partially purified according to Massoulie et al. [1]. A pure globular form (11.8 S) was obtained by trypsinization of D [7]. The enzymatic activity was estimated by Ellman's method [8] and the protein concentration by the Folin technique. We measured absorption at 280 nm of diluted and purified enzymatic solutions, taking an extinction coefficient $\epsilon^{1\%}$ equal to 17.6. The specific activities of injected AchE were 9 mM Asch* /hr/mg for A, 160 mM for C and D, and 50 mM for the globular form (G).

2.2. *Immunization*

0.5 mg of a given antigen solution was mixed with an equal volume of complete Freund's adjuvant and

* Asch: hydrolyzed acetylthiocholine.

North-Holland Publishing Company – Amsterdam

injected intramuscularly into various sites. Rabbits were bled a month after injection, and the more active antisera kept for study.

2.3. *Immunodiffusion*

Detection of antibodies against AchE was made by the double diffusion technique of Ouchterlony [10] in 1.2% ionagar gel (No. 2 Oxoid) and 0.05 M veronal buffer, 1 M NaCl, pH 8.2. The strong ionic strength prevents any aggregation of the elongated forms. Besides already described antigens, we used a commercially available AchE preparation (globular form, from Sigma). AchE activity is revealed by Uriel's technique [11] using indoxylacetate and copper salts. The stain is not specific for AchE. However we checked that our purified preparations did not contain any other type of esterase. We used 1% amidoblack as protein stain.

2.4. *Precipitation tests*

We added increasing amounts of immune serum to a constant concentration of antigen. The residual enzymatic activity was measured after centrifugation, following the technique of Yanofsky [12].

2.5. *Complement fixation*

The microcomplement fixation technique was performed as described by Levine [13]. AchE used for the assay was from highly purified preparations [2], their specific activity being 300 mM/Asch/hr/mg for the D form, 260 for the C and G_t forms. We estimated that D and G_t were at least 95% pure and that C was 80% pure, as shown by acrylamide gel electrophoresis with sodium dodecylsulphate and analytic ultracentrifugation.

2.6. *Hydrolytic enzymes*

Hydrolysis by trypsin results in the formation of the globular form and also of degradation residues [7]. The reaction was stopped by adding a trypsic inhibitor (soja) at a concentration 5-fold that of trypsin. Degradation residues (with sedimentation constants between 0–6 S and 13–25 S) were collected on a sucrose gradient.

2.7. *Pure phospholipase A*

Pure phospholipase A was a gift from Pr. Boquet. Phospholipase C from *Clostridium perfringens* (Worthington) was further purified by DEAE column chromatography. Both hyaluronidase and neuraminidase were Worthington products. The enzymes had been previously tested in order to detect any contaminant protease (using [^{125}I]casein); however, the amount detected was too low to bring any significant conversion of elongated form into globular form.

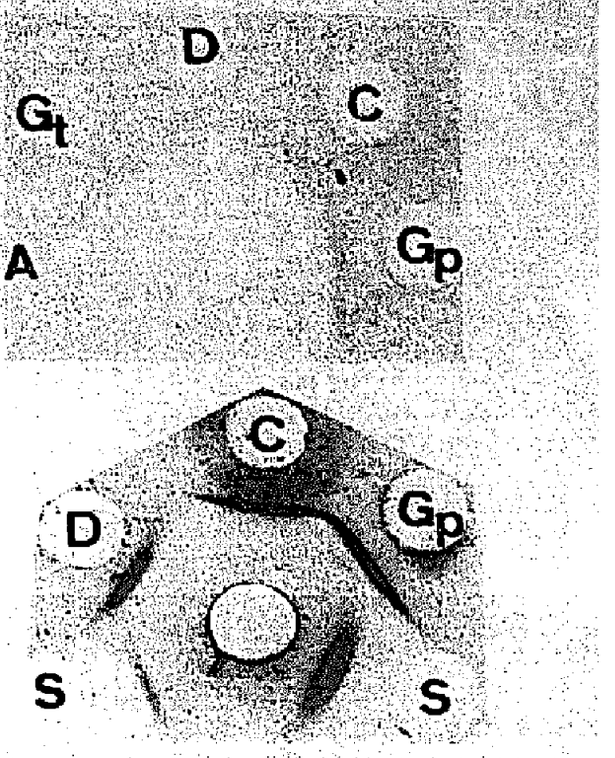

Fig. 1. Immunodiffusions of elongated and globular forms of electric eel acetylcholinesterase against homologous and heterologous antisera. Immunodiffusion made in 1.2% ionogar gel. A) Center well: antiserum 7_G; outside wells: elongated forms, A, C, and D; and globular forms G_t (purified, 11.8 S) and G_p (commercial from Sigma, 11.1 S). Acetylcholinesterase activity: 5 mM Asch/P/ml/hr (10 µl in a well). The single precipitation line was stained by indoxylacetate. B) Center well: antiserum 13_D; outside wells: elongated forms C and D; globular form G_p; and eel serum S. The precipitation line common to D, C and G_p contained all the enzymatic activity, and appeared incurvated in front of the D-containing well. A second (inactive) band can be seen in front of the G_p containing well and is identical to that of a protein found in eel serum.

3. Results

We studied three antisera from rabbits injected with C, D (elongated forms) and G (globular form):

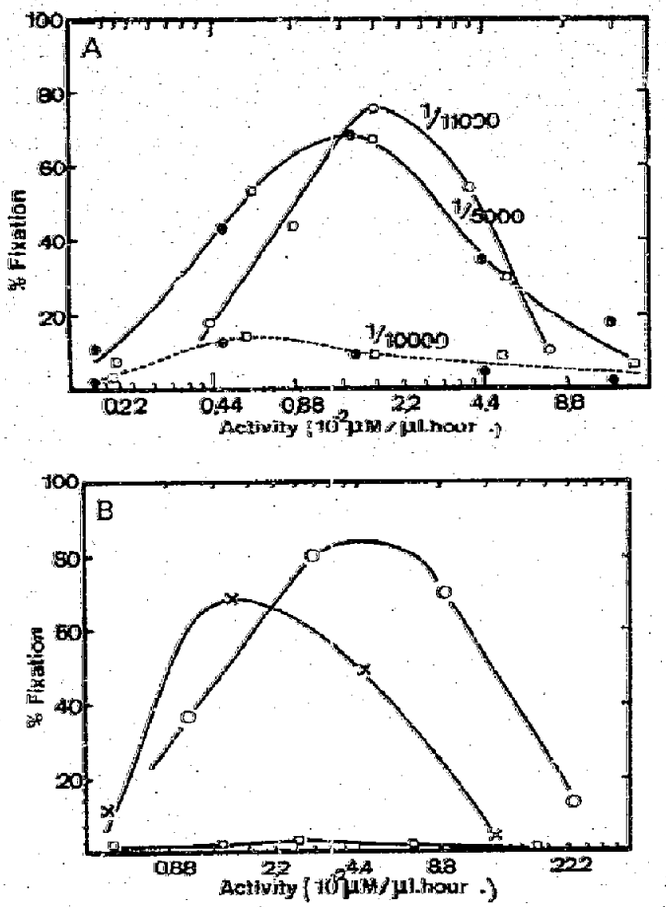

Fig. 2. Complement fixation curves of elongated and globular forms with homologous and heterologous antisera. A) Antiserum 7_G diluted 1/5000; complement fixation curves of globular forms: G_t (□—□—□) and G_p (Sigma) (●—●—●). Antiserum 7_G diluted 1/10000: at this dilution the elongated form D (○—○—○) was detected; the globular forms (□ and ●) did not fix any complement. B) Antiserum 13_D diluted 1/2600; complement fixation curves of elongated form C (×—×—×) and D (○—○—○). At this dilution, the pure globular form G (□—□—□) did not fix any complement.

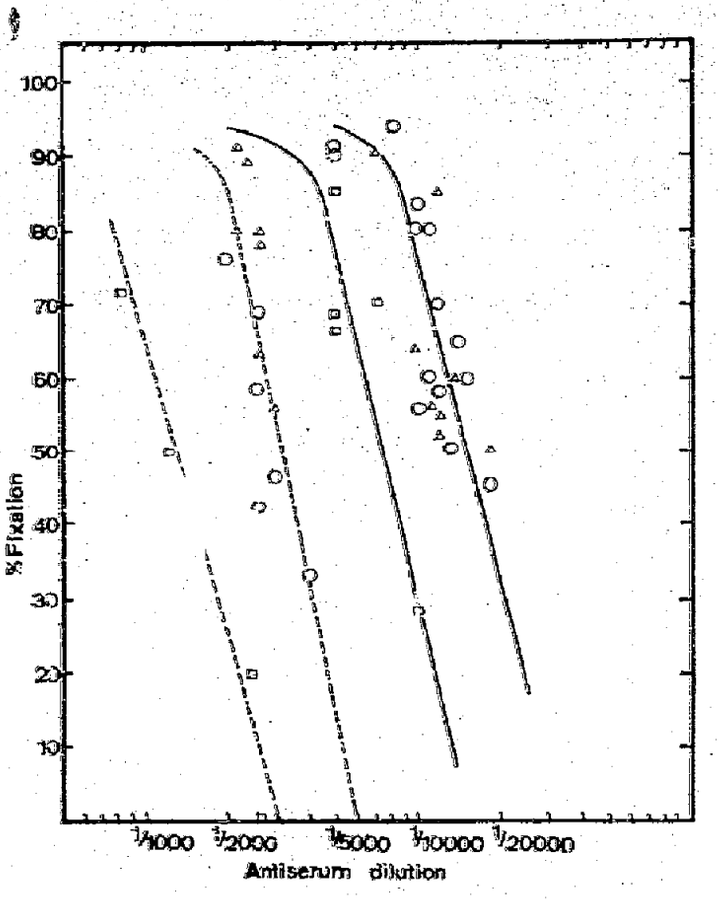

Fig. 3. Complement fixation as a function of antiserum concentrations. A series of dilutions of forms G (□), C (○) and D (△) were tested against several concentrations of antiserum 13_D (----) and antiserum 7_G (——). Each point represents the peak heighth of a complement fixation curve for a given antiserum dilution.

respectively antisera 4_C, 13_D, 7_G. With forms A, C, D and G, each antiserum gave a single precipitation band containing all the enzymatic activity (fig. 1A). When each antigen was tested against the various antisera available and a previously obtained antiserum 968, a similar line of coincidence without spur formation was seen.

Two of the antisera appeared to be monospecific, giving no other band than the AchE one: antiserum 7_G reacted with G form and antiserum 13_D with D and G forms. With D form the antiserum 13_D showed an incurvated precipitation band due to the high molecular weight of D form. With a commercially available G form, this antiserum showed a second band, enzymatically inactive, identical to that given by a protein from eel serum and already observed with antiserum 968 [3], which disappeared after absorption of antiserum 13_D with eel serum (fig. 1B).

Since no difference could be seen in the reactivity of A, C, D and G forms, we attempted to get immunoelectrophoretic data, but the ionic strength necessary to prevent any aggregation of the elongated forms (> 0.3 M) did not give us any satisfactory migrations.

Antisera 13_D and 7_G showed typical complement fixation curves with the three elongated forms and with the globular form as antigens. The zone of antibody–antigen equivalence was defined by reference to the enzymatic activity/ml and not to the protein concentration of the sample. For all the antisera, this

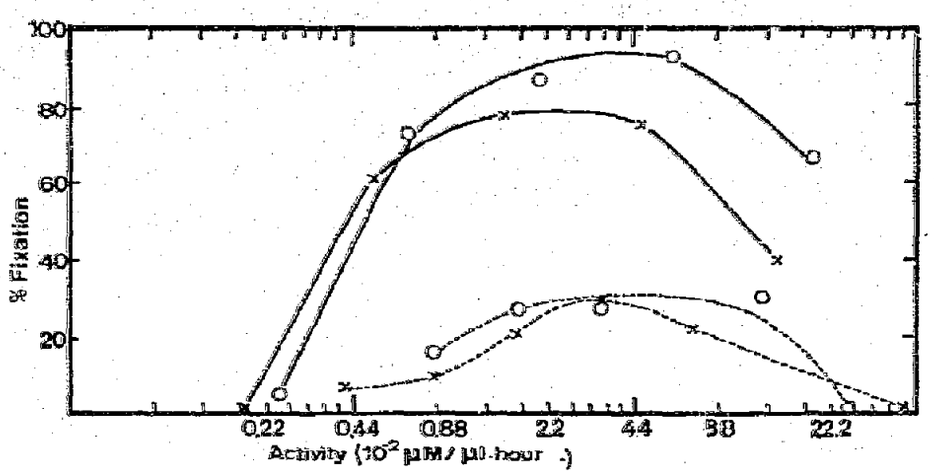

Fig. 4. Competition experiments between G and D, using antiserum 13_D. Antiserum 13_D was diluted at 1/2600; G was added at an activity 10 times higher than D; after 20 min, D (o--o--o) or C (x--x--x) were added. Controls were made with antiserum D (o—o—o) and antiserum C (x—x—x).

zone was observed in an activity range of 2 to 3×10^{-2} mM Asch/hr/ml (fig. 3).

At a 1/5000 dilution of antiserum 7_G, the amount of complement fixed with the homologous antigens was around 70%; at a dilution of 1/10000, the complement was no longer fixed (fig. 3A). However, at such a dilution, the C and D elongated forms reacted with the serum. The same results were obtained with antiserum 968.

With antiserum 13_D, at a dilution of 1/2600, the amount of complement fixed with the homologous C and D forms was 70–80%, whereas there was no fixation with the globular form, unless the concentration of the antiserum was increased by a factor of 2 (fig. 2B). When we plotted the height of the different fixation peaks against the log concentration of the antisera, we found a linear relationship. The slopes of all the lines appeared identical for both the elongated and the globular form: as Sarich and Wilson [14] did when they described similar results in comparing primate albumins, we found the immunological "distance" between D and C to be 1 (since they coincide) and the "distance" between G and D or C to be 2.4 with antiserum 7_G and 2.0 with antiserum 13_D (fig. 3). Thus we demonstrate a higher reactivity of the elongated forms of AchE, whatever antiserum was used, homologous or heterologous.

We made a few attempts to detect antibodies directed solely against the specific determinants of the elongated forms, especially those located in the "tail". We absorbed antiserum 13_D with G form: in immunodiffusion tests, the absorbed antiserum no longer reacted with D form.

We performed competition experiments for a given antiserum between the homologous and heterologous forms: thus G form was added in large excess prior to adding D form. If determinants unique to D were present, one would expect to get a normal or partly depressed complement fixation. We found that some complement was indeed fixed, but that the peak was almost completely flattened. Since, under the conditions used, our control experiments yielded high fixation peaks (greater than 90%) over a large range of antigen concentrations, we estimated that those low peaks did not reflect the presence of antibodies directed against determinants unique to D (fig. 4).

We also assayed polypeptide residues obtained after trypsin digestion of the elongated form and collected on a sucrose gradient. The residues did not fix complement and had no effect in inhibition experiments.

Finally, we investigated the action of hydrolytic enzymes using the complement fixation technique. Grafius et al. [15] recently described the effect of a few lipolytic enzymes on aggregated forms of AchE. Our own data have already shown that treatment with phospholipase C results in some changes in the sedimentation constant [6]. After enzymatic treatment, AchE was again isolated on sucrose gradient. With any of the enzymes tested, complement fixation assays showed no effect on globular forms, nor was

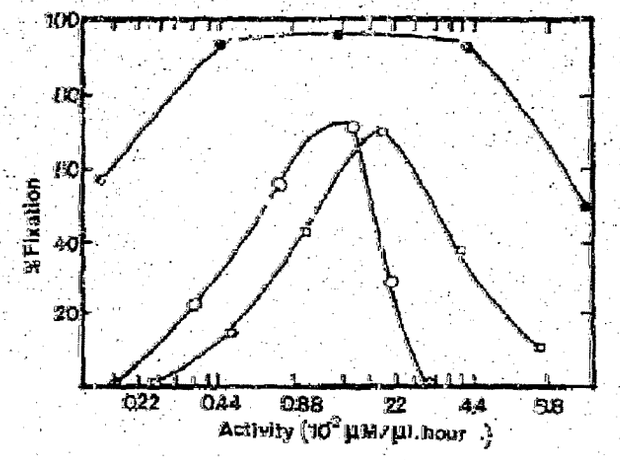

Fig. 5. Effect of phospholipase C hydrolysis of D form on the complement fixation activity. D form was treated by phospholipase C (0.5 mg/ml) for 48 hr, at 20° (o—o—o); D form (•—•—•) and G form (□—□—□) were untreated. Complement fixation curves were obtained with antiserum 7_G (diluted at 1/7000). As can be seen, the curves for treated D and untreated G are identical.

there any effect when elongated forms, such as D, were treated with hyaluronidase or phospholipase A. However, with phospholipase C, the change in sedimentation constant and a striking decrease of the fixation peak (fig. 5) were both observed. The magnitude of these changes suggests that the treated D form was converted into a globular form.

After a neuraminidase treatment of form D, no change was observed in the sedimentation constant, but the complement fixation peak significantly decreased after 2 hr of hydrolysis, and disappeared after 24 hr (fig. 6). A neuraminidase effect was also found on form C, but it appeared slowly and was complete only after 3 hr of hydrolysis.

Thus, complement fixation studies have shown changes in the D and C forms which would not have been detected otherwise (table 1).

4. Discussion

Two anti-AchE sera, one against elongated forms, the other against a globular form, reacted with both homologous and heterologous antigens, giving an identical immunodiffusion reaction with a simple band of precipitation and no spur. In complement fixation tests, the reactivity of A, C and D elongated forms with the homologous and heterologous antisera was greater than that of globular forms, as indicated by a much higher fixation peak. This lower immunological reactivity of the globular form might be related to differences in the overall conformation of the AchE molecule: one could imagine that the globular form (lacking a "tail", or having a reduced one) has been modified in the number or reactivity of its surface determinants ("squeezing" effect). But we cannot rule out the possibility of a conversion *in situ* of the D form injected in the rabbit into a globular form, so that we

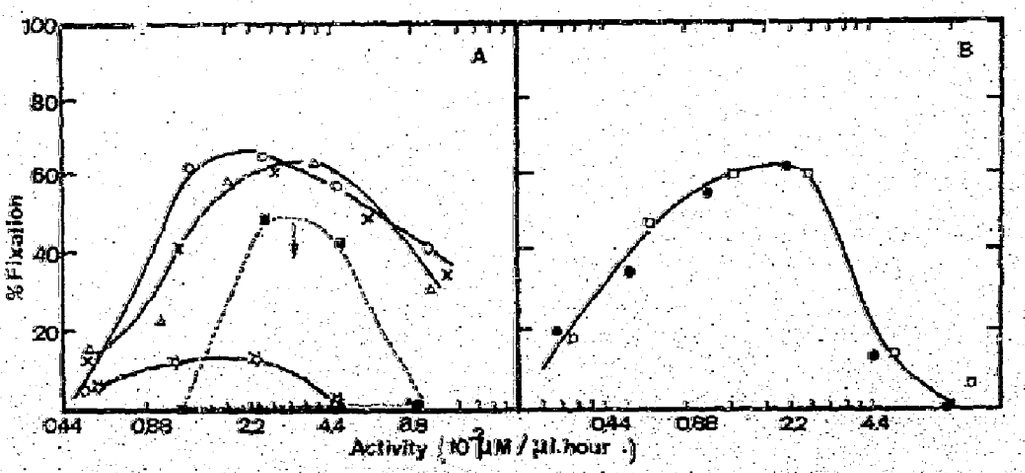

Fig. 6. Effect of neuraminidase hydrolysis of C and D forms on the complement fixation activities. A) Complement fixation curves obtained with antiserum 7_G diluted at 1/11000 and addition of neuraminidase (0.2 mg/ml) at 20°. D form: Control (o—o—o); 2 hr hydrolysis (▪--▪--▪); 24 hr hydrolysis, (▲--▲--▲). C form: Control (x—x—x); 24 hr hydrolysis (▵—▵—▵); 72 hr hydrolysis, (⋆—⋆—⋆). B) Complement fixation curves obtained with antiserum 7_G diluted at 1/5000 and addition of neuraminidase as in A. G form: Control (□—□—□); 72 hr hydrolysis (•—•—•).

Table 1
Effect of various hydrolases on globular and elongated forms of electric eel acetylcholinesterase.

AchE forms	Hyaluronidase or Phospholipase A		Phospholipase C		Neuraminidase				
	S.C.	C'Fix	S.C.	C'Fix	S.C.	C'Fix after			
						2 hr	24 hr	48 hr	72 hr
G	–	–	–	–	–	–	–	–	–
C	–	–	+	++	–	–	–	+	++
D	–	–	+	++	–	+	++	+++	+++

+ Change in sedimentation constant (S.C.) or in complement fixation (C'Fix).
– No change.

Complement fixation was done with antiserum 7_G diluted at 1/5000 when G form was tested; diluted at 1/11000 when C and D forms were tested. Incubation times with hydrolytic enzymes were: 2 hr for phospholipase C; 48 hr for hyaluronidase or phospholipase A; and, as indicated, for neuraminidase.

would actually have been comparing only anti-G sera, containing antibodies with distinct affinities.

Since it has been shown that the elongated forms are made of a "tail" and a "head" [2], we investigated whether specific antibodies might be produced against determinants unique to the "tails". We observed that form G and D were reacting with the same population of antibodies, as shown by inhibition experiments in which a large excess of G almost completely inhibited the complement fixation reaction of antiserum 13_D. Moreover, a close examination of all the fixation curves obtained with the two antisera and the various antigens, shows that all the fixation peaks occur at the same enzymatic concentration.

Nevertheless, with antiserum 4 (from rabbit 4 injected with C form), we observed differences between the various elongated forms: indeed antiserum 4 was not monospecific, but the enzymatic activity corresponding to the zone of equivalence antiserum 4/C was in the range of the activity observed with antiserum 13_D/C and antiserum 7_G/C, and the amount of complement fixed at equivalence was similar for all the curves. However, with antiserum 4, we found a lateral shift of the fixation curves from A to C to D, indicating a decrease in the ratio of antigenic to enzymatic activities, together with an increase of the AchE structure complexity.

The complement fixation technique was sensitive enough to detect structural changes of the elongated forms after treatment with phospholipase C or neuraminidase. With phospholipase C, we observed parallel changes of sedimentation constant and complement fixation curves. After incubation with neuraminidase, although the sedimentation constant was unchanged, we demonstrated a progressive decrease of the affinity of the antiserum for the elongated forms (slower for the form C). Neuraminidase hydrolyzes sialic acid residues. Therefore, sialic acid, and presumably other polysaccharides, give an important contribution to the structural pattern of almost all antigenic determinants of the elongated forms.

We know that all the forms (A, C, D and G) contain sialic residues (ratio 3/1 for D/G) [16], so that there would be a class of sialic acid residues which are present in form G, the hydrolysis of which had no consequences on the antigenicity of this form; and on the other hand, there would probably be another class of sialic acid residues which is necessary for the overall conformation of D form. Further studies on the effect of neuraminidase on A form should indicate whether they are dependent upon the presence of intertetrameric links. In any case, after these links have been broken (by trypsin or ultra-sound action), neuraminidase treatment no longer results in any change of complement fixation.

The significance of the action of phospholipase C – conversion of elongated form into globular form and corresponding complement fixation – seems to be as follows: some phospholipids are necessary to the cohesion of the total structure ("en nappe") of the

D or C form, and these phospholipids are integral parts of the intertetrameric links.

Thus, AchE in its native (D) form is a glycoprotein where some phospholipids are of structural importance for the cohesion of the tetramers of the "head", as well as for the "head"–"tail" interaction.

Acknowledgements

We acknowledge the helpful suggestions of J. Massoulie and S. Bon. We are gratefully indebted to Prof. J.P. Changeux for his comments and critical reading of the manuscript and to H. Costinesco for her help in editing this paper.

References

[1] J. Massoulié, F. Rieger and S. Bon, European J. Biochem. 21 (1971) 542–551.
[2] F. Rieger, S. Bon, J. Massoulié and J. Cartaud, European J. Biochem., in press.
[3] Ph. Benda, S. Tsuji, J. Daussant and J.P. Changeux, Nature 225 (1970) 1149.
[4] S. Tsuji, F. Rieger, G. Pelitre, J. Massoulié and Ph. Benda, J. Neuroch. (1972) 989–997.
[5] W. Leuzinger and A.L. Baker, Proc. Natl. Acad. Sci. U.S. 57 (1967) 446.
[6] F. Rieger, S. Bon and J. Massoulié, European J. Biochem., submitted.
[7] J. Massoulié, F. Rieger and S. Bon, Compt. Rend. 270, serie D (1970) 1837–1840.
[8] G.L. Ellman, K D. Courtney, V. Andres and R.M. Featherstone, Biochem. Pharmacology 7 (1961) 88–95.
[9] Y. Dudai, I. Silman, N. Kalderon and S. Blumberg, Biochim. Biophys. Acta 268 (1972) 138–157.
[10] Ö. Ouchterlony, in: Progress in allergy, ed. P. Kallos (Karger, Basel and New York, 1958) Vol. 5, pp. 1–78.
[11] J. Uriel, Ann. Inst. Pasteur 101 (1961) 104–109.
[12] C. Yanofsky, Ann. N.Y. Acad. Sci. 103 (1963) 1067–1074.
[13] L. Levine and D.M. Weir, in: Handbook of experimental immunology (Blackwell, Oxford, 1967) pp. 707–719.
[14] V.M. Sarich and A.C. Wilson, Science 154 (1966) 1563–1566.
[15] M.A. Grafius, H.A. Bond and D.B. Millar, European J. Biochem. 22 (1971) 382–390.
[16] J. Powell and S. Bon, in preparation.